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14. ABSTRACT
Disease persistence is the main issue faced by CML patients on therapy with imatinib and eradication of persistent malignant cells will be critical for the long-term success of kinase inhibitor therapy. Mechanisms underlying acquired resistance to imatinib have been extensively studied and the manner by which mutations of the Bcr-Abl kinase domain can reduce or eliminate sensitivity of CML cells to imatinib has been well characterized. Disease persistence in responding patients, in contrast,is still poorly understood. We sought to identify and extensively characterize hematopoietic stem cells responsible for disease persistence and explore their mechanisms of imatinib resistance. Using in vitro culture of primary CML progenitor cells, we identified both quiescent and cycling cells capable of surviving in the presence of imatinib. We observed inhibition of tyrosine phosphorylation by imatinib in phenotypically-defined CML stem cells and quiescent stem cells, and cells surviving in vitro culture, suggesting a Bcr-Abl independent mechanism of survival. To apply information gained from in vitro culture to persistent cell populations in treated CML patients, we attempted to isolate Bcr-Abl positive cells from patients in cytogenetic remission. Although persistent CML cells may reside within the stem cell compartment, techniques of stem cell enrichment did not lead to enrichment of CML cells. We therefore explored techniques for Bcr-Abl-specific detection to facilitate these studies, including creation of a Bcr-Abl junction-specific antibody, development of a Bcr-Abl mRNA junction-specific molecular beacon and analysis of potential markers of CML cells. The detailed analysis of primary samples is technically challenging, but is essential for an understanding of disease persistence and may allow identification of novel drug targets or methods to sensitize resistant cells to imatinib or alternative Bcr-Abl kinase inhibitors.

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INTRODUCTION

Targeted therapy with the Abl kinase inhibitor imatinib (Gleevec) induces hematologic and cytogenetic remission in the majority of chronic phase CML[1]. Very few patients, however, have undetectable leukemic cells when more sensitive detection techniques are used and the majority of these patients relapse if imatinib is discontinued[2]. Thus, disease persistence is the main issue faced by the majority of CML patients on therapy with imatinib and eradication of persistent malignant cells will be critical for the long-term success of kinase inhibitor therapy. As Bcr-Abl positive cells persist for up to five years, thus far, this argues that some imatinib-resistant populations are hematopoietic stem cells (HSC) with long-term self-renewal capacity. In fact, $CD34^+$ cells from complete cytogenetic remission (CCR) patients are enriched for $Bcr\text{-}Abl^+$ cells[3]. Many mechanisms of disease persistence have been proposed, including drug efflux[4-8], Bcr-Abl kinase domain mutations[9], Bcr-Abl amplification[10], stem cell quiescence[11,12] and protection by the bone marrow microenvironment[13]. Evidence that these processes apply to persistent cells is limited and mostly circumstantial and thorough analysis of resistance mechanisms in persistent cells from CCR patients has not been done. The goals of this project were to identify and extensively characterize hematopoietic stem cells responsible for disease persistence and explore their mechanisms of imatinib resistance.

The specific aims are: 1) To determine if Bcr-Abl is active in HSC populations that survive imatinib treatment and to determine which mechanisms contribute to the survival of these cells; and 2) To determine which subpopulations of cells are persistently $Bcr\text{-}Abl^+$ in imatinib treated CML patients that have achieved CCR.

BODY

<u>Aim 1</u> – Determine if Bcr-Abl is active in HSC populations that survive imatinib treatment and determine which mechanisms contribute to the survival of these cells

Bcr-Abl is inhibited by imatinib in CML stem cell and progenitor cells

CML $CD34^+$ progenitor cells are sensitive to imatinib inhibition[14], however, the activity and imatinib-sensitivity of Bcr-Abl in more primitive cell types is not known. We wished to determine whether immature cell subtypes as defined by cell surface phenotype were equally sensitive to Bcr-Abl inhibition by imatinib. Intracellular FACS for Bcr-Abl-specific targets P-CrkL, P-Abl and total P-tyr was explored as a means of analyzing small numbers of primary cells. In $CD34^+$ CML cells all three antibodies demonstrated target specificity; however, total phosphotyrosine was chosen for our studies based on superior signal relative to background (Figure 1).

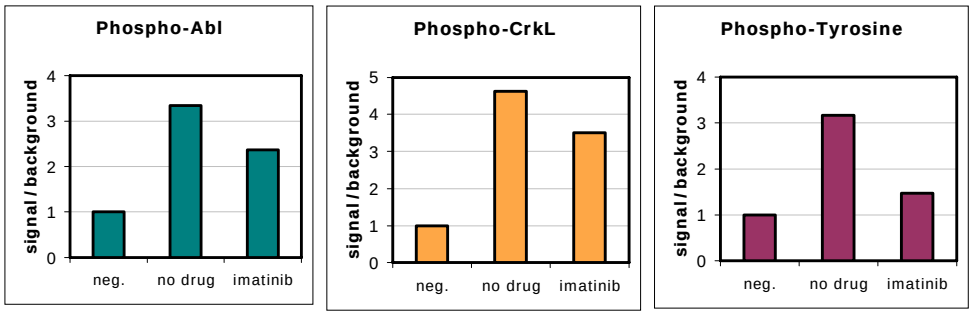


Figure 1. Intracellular FACS analysis of CML CD34+ cells using phospho-Abl (left), phospho-CrkL (middle) and total phosphotyrosine (right) antibodies. Signal relative to background is shown for cells incubated with or without 10μM imatinib.

Phosphotyrosine levels were analyzed by intracellular FACS in lineage marker-depleted (Lin^-) cells from newly diagnosed CML patients and normal bone marrow donors. Untreated cells were compared to those treated briefly with 5 μM imatinib in serum/cytokine-free conditions. Cells were co-stained with CD34, CD38 and CD133 to define stem and progenitor cell subpopulations. As expected, $CD34^+CD38^+$ cells, which primarily represent multipotent committed progenitors, showed a two-fold increase in phosphotyrosine mean fluorescence intensity (MFI) relative to isotype control that was reduced by imatinib treatment (Figure 2A). Although the MFI in imatinib-treated cells was not completely restored to the level of the isotype control, signals in imatinib-treated cells were comparable to normal $CD34^+CD38^+$ cells with or without imatinib suggesting that imatinib inhibition of signal observed in CML cells was Bcr-Abl specific while residual signal was likely background staining. Phenotypic subgroups $CD34^+CD38^-$ and $CD133^+$, representing compartments enriched for stem cell activity, also showed cytokine-independent induction of phosphotyrosine that was reduced to that of normal counterparts by the presence of imatinib (Figure 2A).

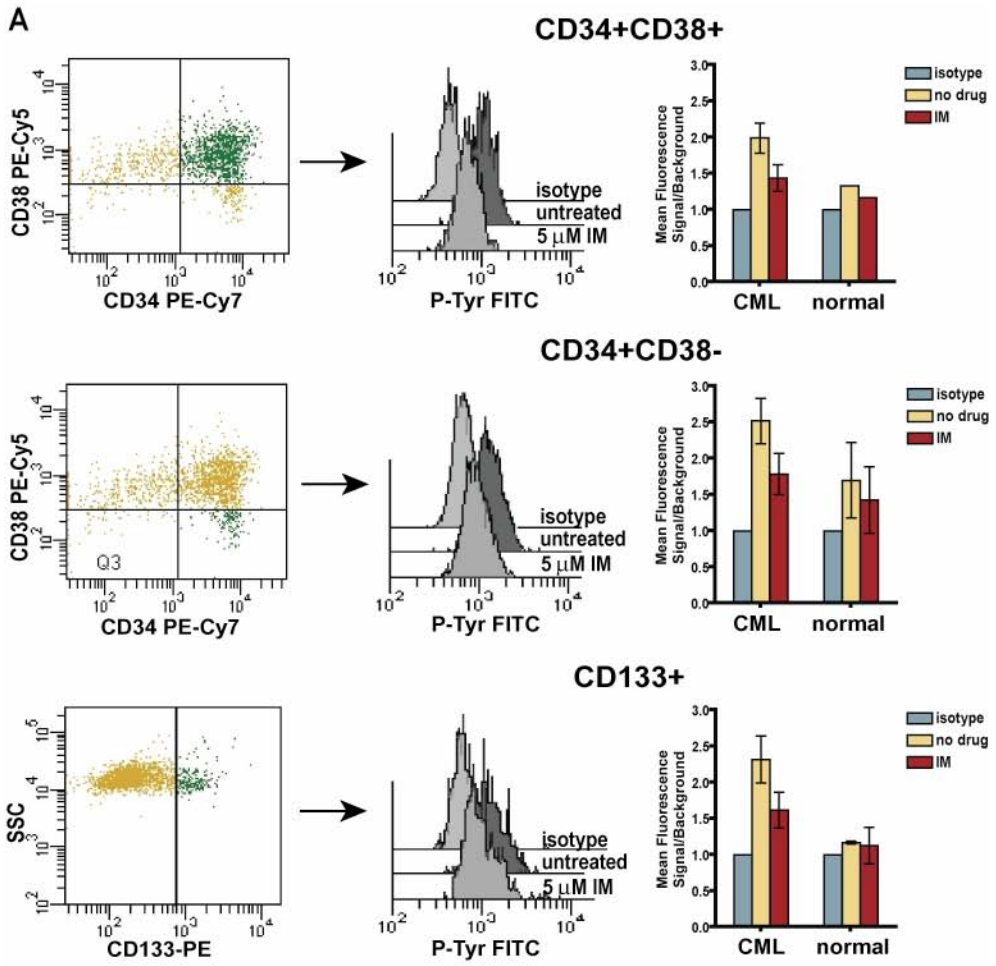
Total phosphotyrosine levels are a general indicator of cell signaling and may not always specifically correlate with Bcr-Abl activity. We therefore examined the phosphorylation status of the Bcr-Abl-specific substrate CrkL in $CD34^+CD38^-$ stem cells and $CD34^+CD38^+$ progenitor cells. We performed P-CrkL immunoblot analysis on FACS sorted stem and progenitor cell populations. Consistent with the results observed for total cellular phosphorylation, phospho-CrkL levels were completely inhibited by 5 μM imatinib in both $CD34^+CD38^-$ and $CD34^+CD38^+$ cells (Figure 2B). The results of the two complementary assays demonstrate that Bcr-Abl kinase activity is sensitive to the effects of imatinib in both stem and progenitor cell populations.

Cytokine supplementation supports in vitro proliferation of CML Lin^- cells despite continuous imatinib inhibition of Bcr-Abl

We next evaluated how inhibition of Bcr-Abl activity within CML Lin^- cells affected their capacity to survive and proliferate. For these studies, bone marrow and leukapheresis samples from seven newly diagnosed, imatinib-naïve chronic phase CML patients and two normal samples were used. Mononuclear cells, isolated by Ficoll centrifugation, were depleted, by immunomagnetic separation, of cells expressing lineage markers. Lin^- cells were cultured in serum free medium either without cytokines or with a five cytokine cocktail (100ng/mL Flt ligand, 100ng/mL SCF, 20ng/mL IL-3, 20ng/mL IL-6, 20ng/mL G-CSF) in the presence of imatinib for four days as described[12]. Previous studies have indicated CML $CD34^+$ cells are capable of cytokine-independent growth that is inhibited by imatinib[12]. We also found that short-term in vitro proliferation of Lin^- CML cells in the absence of cytokines, serum and stromal support was dependent on Bcr-Abl activity (Figure 3A).

Although some CML cells could survive short term culture in imatinib, similar survival rates were seen with normal progenitor cells under the same culture conditions, suggesting that, in the absence of external supportive factors, CML progenitors require Bcr-Abl activity to thrive. In the presence of cytokines, CML cells demonstrated a proliferative advantage relative to normal cells that was suppressed by imatinib (Figure 3B). Untreated CML Lin^- cells expanded 9.4-fold on average while imatinib-treated CML cells and imatinib-treated normal cells expanded 4.0 and 4.8-fold respectively with no significant difference between the latter two populations ($p=0.14$). Consistent with data from Holtz et al[15], we observed only a modest increase in apoptosis in imatinib-treated cultures (Figure 3B) suggesting that the reduced expansion capacity in the presence of imatinib was due primarily to suppression of enhanced proliferation, rather than induction of apoptosis. This was additionally confirmed by the observation that cell cycle distribution was identical in untreated and imatinib-treated cultures (Figure 3B). FISH for *bcr-abl* following the culture period demonstrated that, in all

samples >95% of cells were Ph^+ independent of the presence of imatinib, therefore imatinib did not select normal progenitors.



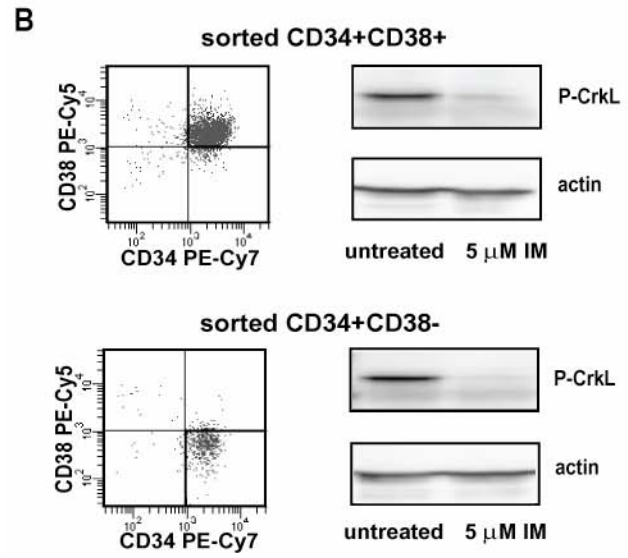


Figure 2. Inhibition of Bcr-Abl kinase activity in CML stem and progenitor cells.
A. Intracellular phosphotyrosine levels were evaluated by FACS in Lin- CML cells co-stained for CD34, CD38 and CD133. Fluorescence intensities for phosphotyrosine signal of treated and untreated cells relative to isotype are shown for CML and normal. **B**. Phospho-CrkL immunoblots with lysate from sorted $CD34^+CD38^+$ and $CD34^+CD38^-$ CML cells treated briefly with or without imatinib demonstrate inhibition of Bcr-Abl by imatinib in both populations. B-actin is shown as a loading control.

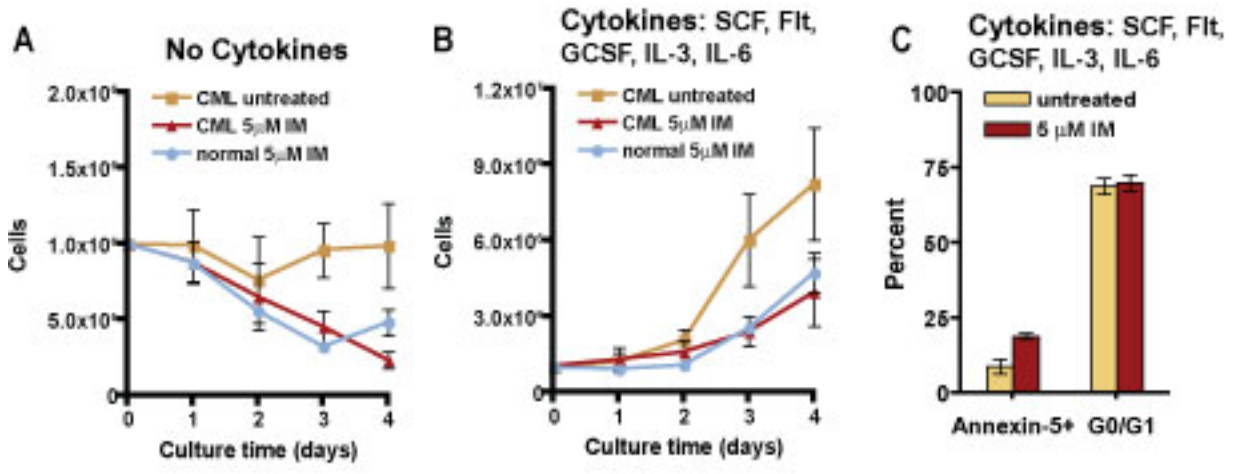


Figure 3. Proliferation and survival of CML progenitor cells in short term imatinib culture.
Cell numbers of newly diagnosed CML or normal Lin$^-$ cells with or without imatinib were monitored daily during a four day culture in **A**. the absence of cytokines **B**. a cytokine cocktail that included SCF, Flt ligand, GCSF, IL-3 and IL6. Apoptosis and cell cycle progression in untreated versus imatinib cultures were evaluated by Annexin-5 and acridine orange staining respectively.

To determine whether Bcr-Abl inhibition was continuous throughout the culture period or imatinib selected a population of resistant cells, we evaluated phosphotyrosine and phospho-CrkL levels following culture. Western blot analysis of phospho-CrkL and total phosphotyrosine (Figure 4A) as well as total phosphotyrosine FACS (Figure 4B) were done on cells from bulk untreated cultures, imatinib treated cultures, and imatinib treated cultures with imatinib washed out (washout). Both assays showed a reduction of Bcr-Abl activity in the imatinib-treated cultures that was restored upon removal of imatinib.

These results indicate that Bcr-Abl activity stimulates excessive growth of CML progenitors with or without cytokines. Imatinib inhibition, however, appears to restore normal homeostatic properties and thus cytokine supplementation may bypass the requirement for Bcr-Abl activity for survival and proliferation of CML progenitors.

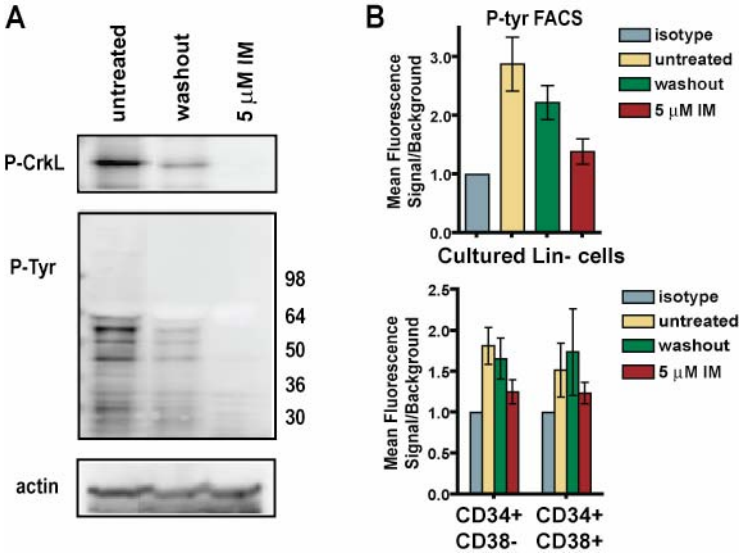


Figure 4. Inhibition of Bcr-Abl activity in cultured CML stem and progenitor cells.
A Phospho-CrkL and total phospho-tyrosine levels in bulk cultured Lin$^-$ cells were analyzed by immunoblot. Untreated cells, 5 μM imatinib-treated cells in which imatinib was washed out prior to immunoblots (washout) and 5 μM imatinib-treated cells are shown from a representative sample. **B** Phospho-tyrosine levels were analyzed by intracellular FACS analysis in bulk cultured Lin$^-$ cells (top) and CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ cells

Cytokine supplementation supports in vitro proliferation and differentiation of CML stem cells despite continuous imatinib inhibition of Bcr-Abl

We next wished to evaluate which phenotypic subgroups were contributing to the Bcr-Abl-independent cell expansion. Phenotypic analysis of lineage-specific markers and CD34 and CD38 expression prior to and following the four-day culture period demonstrated an overall reduction in the frequency of Lin^- and $CD34^+$ cells indicating that the culture conditions were permissive of differentiation (Figure 5A). The phenotypic distribution in untreated versus imatinib-treated cultures was similar, indicating that imatinib treatment did not select for a uniformly resistant subtype. Interestingly, when compared with normal progenitors, CML cultures showed a preferential retention of cells with more primitive phenotypes, suggesting that differentiation response to cytokine stimulation was altered independent of Bcr-Abl activity. There was a slight, but not significant, tendency toward enhanced differentiation in imatinib-treated cultures, which may indicate a shift toward normalized response to cytokines. Untreated CML cells showed enhanced expansion of both stem and progenitor cell compartments relative to normal cells that was restored by the addition of imatinib. Interestingly, absolute numbers of $CD34^+$ cells and $CD34^+CD38^-$ cells both increased in the presence of imatinib in all CML samples evaluated (Figure 5B), however, the degree of expansion was variable between patients (Figure 5C). We additionally used Acridine Orange staining to identify quiescent cells capable of retaining their quiescent phenotype despite the presence of cytokines. The absolute number of quiescent cells following culture was the same in untreated and imatinib treated cells (Figure 5A,B). These results indicate that both quiescent and proliferating primitive CML cells can survive in vitro imatinib treatment.

We analyzed phosphotyrosine levels of $CD34^+$ and $CD34^+CD38^-$ cells following imatinib treatment. As in the bulk culture, we found that phosphotyrosine levels in stem and progenitor cell subtypes were sensitive to imatinib (Figure 4B).

Bcr-Abl is inhibited by imatinib in primitive, quiescent CML cells

CML stem cell quiescence has been associated with imatinib resistance in vitro, leading to the hypothesis that residual disease is comprised of drug-resistant quiescent cells[11,12]. The role of Bcr-Abl in mediating resistance of quiescent cells is unclear. We therefore analyzed Bcr-Abl activity and imatinib sensitivity of quiescent CML cells. Ki-67 expression reliably correlates with all stages of the cell cycle and the absence of this marker is indicative of cell quiescence[16]. We therefore used intracellular FACS to sort Ki-67^- Lin^- and Ki-67^+ Lin^- cells from a newly diagnosed CML sample treated briefly with or without 5 μM imatinib. Although sort purities were >85% by FACS analysis (Figure 6A), we additionally confirmed that the sorted cells showed patterns of protein expression consistent with quiescent or cycling cells. Ki-67 and PCNA immunoblots of sorted Ki-67^+ and Ki-67^- cells showed expression of these markers in the former and lack of expression in the latter, thus verifying that these cells represented cycling and quiescent cells respectively (Figure 6B). Phospho-CrkL immunoblots of the sorted quiescent and cycling cells showed complete inhibition of Bcr-Abl activity by imatinib that was independent of cell cycle status (Figure 6C). These data demonstrate that any innate resistance exhibited by quiescent leukemic cells is unlikely to be Bcr-Abl-dependent.

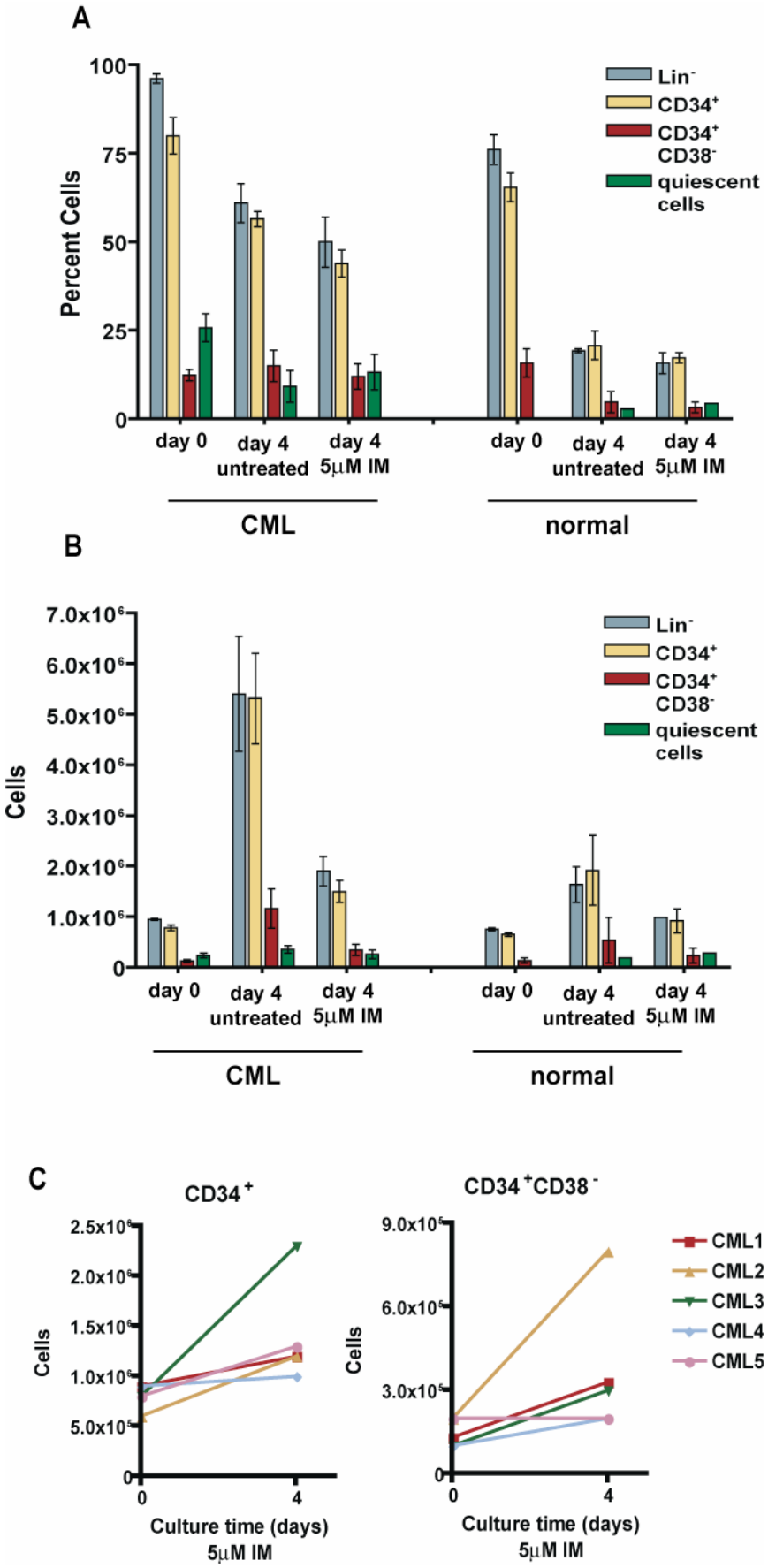


Figure 5. Expansion of different CML stem and progenitor cell phenotypic sub-types following culture with imatinib.

A Relative frequencies of Lin^-, $CD34^+$ and $CD34^+CD38^-$ and quiescent cells prior to and following four day CML Lin^- cell culture. **B** Absolute cell numbers (normalized relative to starting cell density) of Lin^-, $CD34^+$ and $CD34^+CD38^-$ and quiescent cells prior to and following culture with or without imatinib. **C** Expansion of $CD34^+$ and $CD34^+CD38^-$ CML cell numbers in the presence of imatinib shown for individual patient samples.

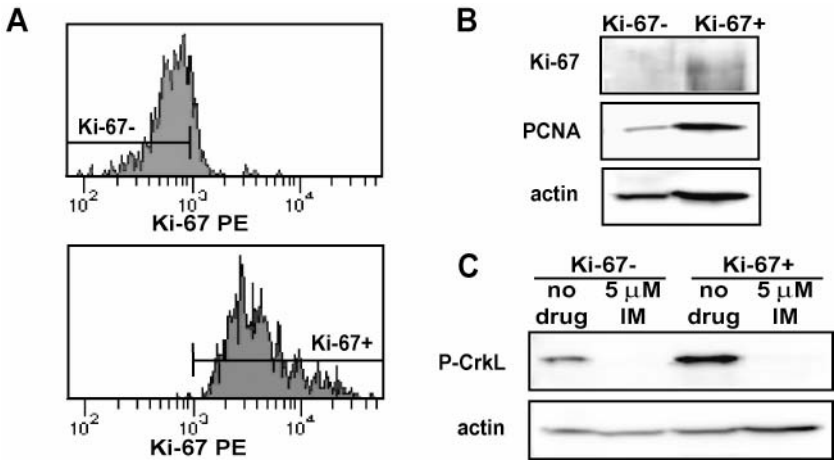


Figure 6. Inhibition of Bcr-Abl kinase activity in quiescent and cycling CML cells.
A. Lin$^-$ Ki-67$^-$ and Ki-67$^+$ CML cells were FACS sorted into quiescent and cycling fractions respectively. **B**. Immunoblots of Ki-67 and PCNA in sorted Ki-67$^-$ and Ki-67$^+$ cells were used to confirm sort purity, as indicated by exclusive expression of these proteins in the cycling fraction. β-actin is shown as a loading control. **C**. Phospho-CrkL immunoblots of quiescent versus cycling cells treated briefly with or without imatinib demonstrate inhibition of Bcr-Abl by imatinib in both fractions. β-Actin is shown as a loading control.

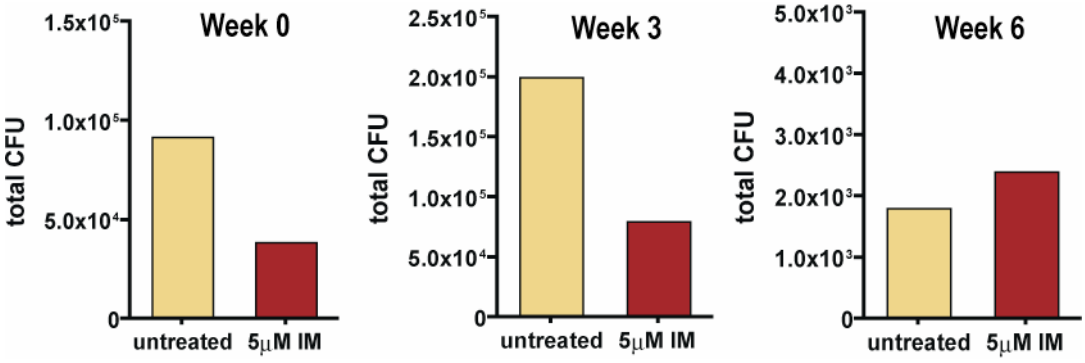


Figure 7. Frequency of CML CFC and LTC-IC following culture with imatinib.
Following four day culture with or without imatinib, week 0 CFU and week 3 and 6 LTC-IC were assessed.

CML stem and progenitor cells survive Bcr-Abl inhibition by dasatinib and nilotinib

Given that in vitro survival and proliferation of CML stem and progenitor cells could occur in the absence of Bcr-Abl kinase activity, we reasoned that dasatinib and nilotinib exert similar effects as imatinib. During a four-day culture in cytokine-enriched media we observed inhibition of proliferation by nilotinib and dasatinib comparable to imatinib. All three inhibitors additionally permitted equivalent cell expansion (Figure 8A). Additionally, phosphotyrosine levels were inhibited by dasatinib and nilotinib in $CD34^+CD38^-$ and $CD34^+CD38^+$ cells following in vitro culture (Figure 8B).

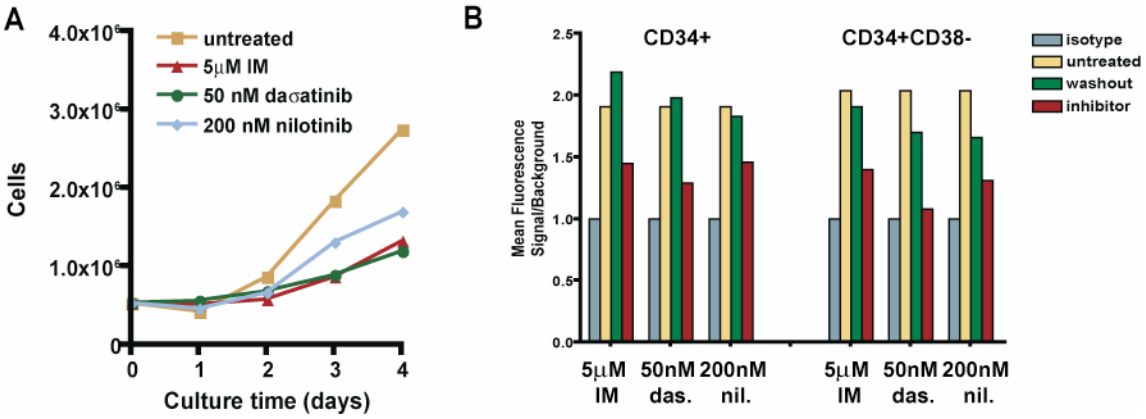


Figure 8 Sensitivity of CML stem and progenitor cells to imatinib, dasatinib and nilotinib.
A. Lin^- CML cells were cultured for four days with SCF, Flt ligand, GCSF, IL-3 and IL-6 in the presence of imatinib, dasatinib and nilotinib. Daily cell counts were used to compare cell proliferation for each inhibitor. **B**. Bcr-Abl activity following culture with the three inhibitors was determined by phospho-tyrosine FACS in $CD34^+$ and $CD34^+CD38^-$ cells.

Aim 2 – Determine which subpopulations of cells are persistently Bcr-Abl$^+$ in imatinib treated CML patients that have achieved CCR

Seeking persistent cells using stem cell enrichment techniques

While our i*n vitro* culture studies provided valuable information regarding Bcr-Abl sensitivity to imatinib in primitive CML cells, it is still unclear whether these cells represent persistent cells in imatinib treated patients. We additionally attempted to study persistent cells in patients who had achieved a complete cytogenetic remission without a molecular remission. Initially, we determined whether enriching stem/progenitor cells would also enrich residual Ph^+ cells as was previously suggested[3]. Bone marrow from CCR patients was lineage depleted and FACS sorted into $CD34^+CD38^-$, $CD34^+CD38^{low}$, $CD34^+CD38^+$ and Lin^+ fractions and analyzed by FISH for the presence of Bcr-Abl. In two samples, 1% Ph^+ cells were seen in the $CD34^+CD38^{low}$ fraction (Table 1); however, this was not sufficient enrichment to pursue this method.

Table 1. Analysis of Bcr-Abl$^+$ cells in stem/progenitor cell fractions of CCR patients.

sample ID	34^+38^- %Ph^+	$34^+ 38^{low}$ %Ph^+	34^+38^+ %Ph^+	Lin^+ %Ph^+
CCR1	0	-	0	-
CCR2	0	1	0	0
CCR3	0	1	0	0
CCR4	0	0	0	0

Because our study and previous studies demonstrated survival of primitive quiescent Bcr-Abl$^+$ cells in the presence of imatinib[12], we additionally analyzed Lin$^-$G$_0$ cells from CCR patients. No enrichment of Bcr-Abl$^+$ cells was observed in the primitive quiescent fraction (Table 2). We concluded that methods of stem cell isolation were unlikely to enrich Bcr-Abl positive cells sufficiently for our studies.

Enrichment of Bcr-Abl-expressing cells

We next sought to refine our methods of Bcr-Abl$^+$ cell enrichment by developing Bcr-Abl-specific means of cell separation. We simultaneously initiated multiple approaches, including detection of Bcr-Abl protein, detection of Bcr-Abl mRNA and identification of CML progenitor cell-specific markers.

Table 2. Analysis of Bcr-Abl$^+$ cells in quiescent stem/ progenitor cells of CCR patients.

sample ID	% G$_0$	%Ph$^+$ G$_0$	%Ph$^+$ "not G$_0$"
CCR5	65	0	0
CCR6	31	5.7	5.9
CCR7	22	0	0
CCR8	53	0	0
CCR9	60	5.4	0
CCR10	46	0	0

Bcr-Abl junction-specific antibodies

To detect Bcr-Abl protein independently of c-Bcr and c-Abl, we generated antibodies in chickens (Aves Laboratory) specific to the Bcr-Abl junction regions b3a2 and b2a2[17]. Antibody target recognition was validated by immunoprecipitation (Figure 9A) and flow cytometry (Figure 9B). The b3a2 antibody was capable of immunopreciptating Bcr-Abl and demonstrated a statistically significant increase in fluorescence signal by FACS in Bcr-Abl b3a2-expressing cells relative to Bcr-Abl negative and Bcr-Abl b2a2 expressing cells. Specificity was not seen with the b2a2 antibody.

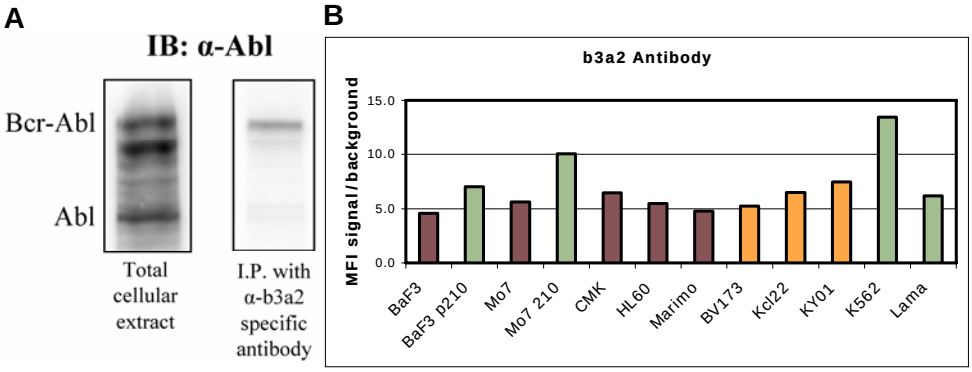


Figure 9. Detection of Bcr-Abl using a b3a2 junction-specific antibody. **A)** Immunoprecipitation of Bcr-Abl with a b3a2 antibody. **B)** Intracellular FACS with a b3a2 antibody in Bcr-Abl negative (purple) b3a2 (green) and b2a2 (orange) Bcr-Abl expressing cells.

K562 cells diluted into Bcr-Abl-negative HL60 cells were sorted based on the b3a2 antibody signal and sorted populations were analyzed by FISH for Bcr-Abl. Enrichment of Bcr-Abl$^+$ cells was seen even in a high background of Bcr-Abl$^-$ cells (Table 3).

Table 3. Enrichment of Bcr-Abl$^+$ cells with a Bcr-Abl b3a2-specific antibody

Dilution	Post Sort b3a2$^-$ %Ph$^+$	Post Sort b3a2$^+$ %Ph$^+$
1:1 K562:HL60	1%	100%
1:100 K562:HL60	0%	72%

CML CD34$^+$ and normal CD34$^+$ bone marrow cells were analyzed by intracellular FACS with the b3a2 antibody. While initial selective detection of CML cells was observed in a single CML patient, (Figure 10A) this result was not consistent across multiple samples and no statistical significance in FI between normal and CML samples was seen (p=0.08).

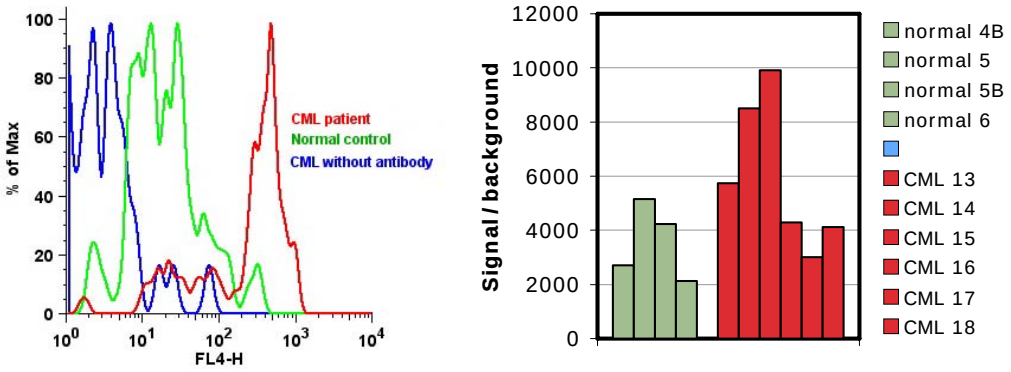


Figure 10. Intracellular FACS analysis of normal (green) and CML (red) CD34+ cells with a b3a2 specific Bcr-Abl antibody.

Because this is a polyclonal antibody, high background staining may obscure weaker differences in signal. To address this issue, we employed methods of subtractive affinity purification to remove any contaminating antibodies that may bind to c-Bcr or c-Abl. Additionally, we used subtractive affinity purification with Mo7 lysate to remove antibodies that were binding to non-Bcr-Abl cellular proteins. Although background staining was reduced following additional steps of purification, neither method was able to improve specific recognition of Bcr-Abl-expressing cells (data not shown).

Bcr-Abl mRNA hybridization

To specifically detect Bcr-Abl mRNA, we designed a single stranded DNA probe against the Bcr-Abl b3a2 junction. A molecular beacon dye/quencher strategy was used as a method of signal detection[18]. Bcr-Abl specific signal was observed in K562 cells with the molecular beacon relative to a non-specific scrambled probe (Figure 11) however background signal was high, even under optimized hybridization conditions.

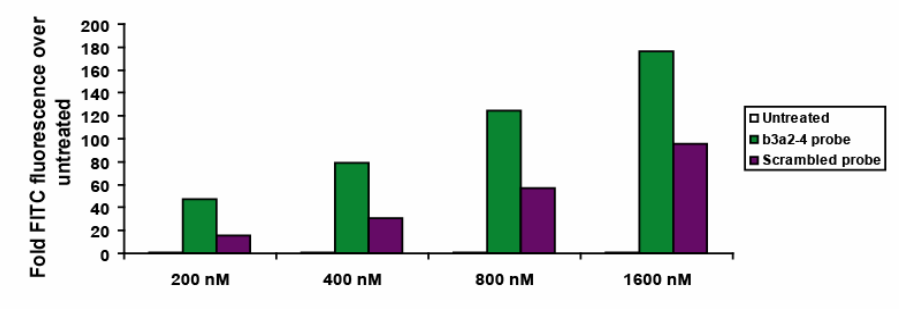


Figure 11. Detection of Bcr-Abl in K562 using a b3a2 junction-specific molecular beacon (green) versus a non-specific scrambled probe (purple).

CML-specific markers

An alternative strategy to isolate CML cells from a normal background is to identify markers that are specific for CML stem and progenitor cells. Literature searches identified CD33[19,20], CD123[20,21] and WT-1[22] as candidate markers that were shown to be upregulated either at the mRNA or protein level in CML versus normal cells. Additionally, we performed a microarray meta-analysis using publicly available data of gene expression profiles in normal versus CML $CD34^+$ cells[23] as well as CML $CD34^+$ data that we generated in the context of a separate project. Several candidate cell-surface as well as intracellular markers were identified (Table 4).

Table 4. FACS analysis of candidate markers for CML and normal stem/progenitor cells.

gene	mRNA CML/normal	FACS CML/normal	p value
Leptin Receptor	3.9	1.5	0.14
CD29	3.1	1.1	0.23
CD114	0.30	1.3	0.19
CD61	0.23	1.6	0.14
CD54*	0.20	1.3	0.004
CD33	n/a	2.7	0.12
CD123	n/a	1.0	0.50
Prefoldin-4*	10.1	1.5	0.007
Ski*	9.2	1.4	0.03
K-Ras*	6.7	1.3	0.04
RALA	5.4	1.1	0.13
Opioid receptor mu1	4.4	1.1	0.36
Jak2	4.3	1.2	0.14
TRF1	4.3	1.5	0.06
WT-1*	n/a	1.3	0.008
c-Abl/Bcr-Abl*	n/a	1.6	0.00007
c-Bcr/Bcr-Abl	n/a	1.0	0.38

Abl and Bcr expression levels relative to Bcr-Abl were also considered. All candidate markers were analyzed by FACS using cell surface or intracellular staining of CML and normal CD34+ cells. Relative fluorescence intensities of CML/normal were compared (Table 4). Representative data for WT-1 is shown (Figure 12).

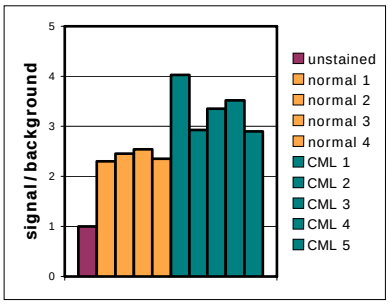


Figure 12. Intracellular FACS analysis of WT-1 expression in normal (orange) and CML (teal) CD34+ cells.

Those demonstrating a statistically different signal between normal and CML (represented by *) all showed <2-fold difference in CML/normal. Additional optimization steps were taken to determine whether the CML-specific signal could be enhanced. Among the difficulties encountered were limited availability of suitable monoclonal antibodies, poor antibody quality, poor antibody specificity and the need to optimize cell permeabilization techniques for intracellular staining. Western blots of a variety of Bcr-Abl negative and positive cell lines demonstrated that the antibody for prefoldin 4 was unable to recognize its target and available antibodies for K-Ras recognize all of the Ras isoforms. Thus, these targets were discarded. Ski-1 and TRF-1 antibodies both showed specificity for their targets in western blots and by FACS analysis, however, the optimal permeabilization technique for FACS was incompatible with cell surface staining for CD34+ cells.

WT-1 expression and c-Abl/Bcr-Abl expression were used as a means of sorting CML from normal cells. CML and normal $CD34^+$ cells stained with either WT-1 antibody or Abl 24-21 were mixed at equal ratios and sorted based on WT-1 or Abl signal.

Table 5. Enrichment of Bcr-Abl^+ cells in WT-1^{high} and Abl^{high} fractions of mixed normal and CML $CD34^+$ cells.

	Post Sort Ph+
WT-1^{low}	38%
WT-1^{high}	78%
Abl^{low}	25%
Abl^{high}	75%

Sorted cells were analyzed by FISH for Bcr-Abl. Incomplete enrichment was observed for both markers tested (Table 5). Additionally, Abl staining and FACS sorting of $CD34^+$ marrow cells from CML patients who had achieved a partial cytogenetic remission on imatinib did not yield any enrichment of Bcr-Abl^+ cells (data not shown) suggesting that the small differences in signal observed with WT-1 and Abl staining may not be sufficient to achieve the separation necessary for our studies.

KEY RESEARCH ACCOMPLISHMENTS

- Analysis of Bcr-Abl inhibition by imatinib in the context of phenotypically-defined CML stem and progenitor cells
- Analysis of Bcr-Abl inhibition by imatinib in quiescent CML progenitors
- Analysis of proliferation, cell cycle status, tyrosine phosphorylation, imatinib sensitivity and stem cell function of newly diagnosed CML stem/progenitor cells in an *in vitro* culture system
- Evaluation of dasatinib and nilotinib sensitivity in the context of CML stem and progenitor cells
- Evaluation of a Bcr-Abl junction-specific antibody to distinguish Bcr-Abl positive CML cells from normal cells
- Evaluation of a Bcr-Abl junction-specific molecular beacon to distinguishing Bcr-Abl positive cells from Bcr-Abl negative cells
- Evaluation of seventeen potential markers for CML stem cells
- Assessment of the frequency of CML cells among stem cell and quiescent progenitor cell populations of patients in CCR

REPORTABLE OUTCOMES

Manuscript in preparation: CML Stem Cells are Insensitive to Imatinib Despite Inhibition of Bcr-Abl Activity. Amie S. Corbin, Michael W.N Deininger, Brian J. Druker.

CONCLUSIONS

To address the mechanisms of disease persistence in imatinib-treated CML, we initially studied the *in vitro* effects of imatinib on newly diagnosed CML stem/progenitor cells. We utilized complementary strategies of FACS sorting, intracellular staining and immunoblots to evaluate imatinib-induced changes in phospho-CrkL and phospho-tyrosine levels in CML cells with stem and progenitor cell phenotypes. $CD34^+CD38^-$ and $CD133^+$ cells are the most phenotypically primitive hematopoietic cells and are greatly enriched for stem cell function as shown previously by LTC-IC and murine long-term engraftment assays[24-26]. Newly diagnosed CML patients showed a reduction of phospho-CrkL and total phosphotyrosine in the presence of imatinib in both of these phenotypically-defined stem cell compartments. The degree of Bcr-Abl inhibition was comparable to more mature $CD34^+CD38^+$ cells.

Previously, it was unknown how Bcr-Abl activity in quiescent cells was affected by the presence of imatinib. We demonstrate that Bcr-Abl activity in primitive CML cells is sensitive to imatinib inhibition independent of cell cycle status. This indicates that any innate properties of quiescent cells that confer resistance to imatinib are not Bcr-Abl-dependent.

We explored how loss of Bcr-Abl activity in primitive CML cells would impact their survival. When exogenous cytokines were supplied, Lin^-, $CD34^+$ and $CD34^+CD38^-$ cells were capable of in vitro proliferation in the presence of imatinib with no disruption of cell cycle and only moderate apoptosis despite continuous inhibition of Bcr-Abl activity. Proliferation of all progenitor cell types was inhibited by imatinib; however, growth capacity in the presence of imatinib was similar to normal progenitor cells. Additionally, quiescent and LTC-IC cell numbers following culture did not appear to be affected by the presence of imatinib despite Bcr-Abl inhibition. Survival of CML stem and progenitor cells and restoration of normal homeostasis by the presence of imatinib suggests that primitive CML cells do not require Bcr-Abl activity for survival when other supportive factors are

present. This finding is corroborated by our observation of in vitro survival of nilotinib and dasatinib-treated CML stem cells. CML stem cell eradication may therefore require alternative strategies such as targeting stem cell function or disrupting interactions with the microenvironment.

To better characterize how imatinib functions in the context of persistent disease, we attempted to isolate persistent cells from patients in cytogenetic remission. Enrichment strategies using stem cell markers or the property of quiescence did not enrich the Bcr-Abl$^+$ population, therefore we focused extensively on methods of Bcr-Abl-specific detection. We generated a Bcr-Abl b3a2 junction-specific antibody, however selective recognition of Bcr-Abl expressing cell lines and primary CML progenitors by FACS analysis was limited. We additionally developed a Bcr-Abl b3a2 junction-specific molecular beacon capable of selectively detecting Bcr-Abl mRNA in Bcr-Abl expressing cells, however, high background staining limited its utility. Finally, we evaluated CML progenitor cell markers identified by microarray meta-analysis, however, no consistently successful marker specific for CML cells was observed.

Despite the difficulties we encountered in our attempts to isolate persistent CML cells in CCR patients, our surrogate in vitro culture assays provided valuable information regarding the sensitivity of Bcr-Abl to imatinib in CML stem cells, the most likely candidate cells directing disease persistence. Our observation that Bcr-Abl kinase activity is sensitive to imatinib in the context of CML stem cells and that these cells are capable of survival and proliferation despite continuous Bcr-Abl inhibition is a novel finding and suggests that the most primitive CML cells are not oncogene addicted. These results are an important step towards understanding how to approach the problem of disease eradication and suggest that Bcr-Abl targeted therapy alone will not be sufficient to eliminate CML stem cells.

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